

## *Bacillus cereus* G9241 Makes Anthrax Toxin and Capsule like Highly Virulent *B. anthracis* Ames but Behaves like Attenuated Toxigenic Nonencapsulated *B. anthracis* Sterne in Rabbits and Mice<sup>▽</sup>

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*Bacillus cereus* G9241 was isolated from a welder with a pulmonary anthrax-like illness. The organism contains two megaplasms, pBCXO1 and pBC218. These plasmids are analogous to the *Bacillus anthracis* Ames plasmids pXO1 and pXO2 that encode anthrax toxins and capsule, respectively. Here we evaluated the virulence of *B. cereus* G9241 as well as the contributions of pBCXO1 and pBC218 to virulence. *B. cereus* G9241 was avirulent in New Zealand rabbits after subcutaneous inoculation and attenuated 100-fold compared to the published 50% lethal dose (LD<sub>50</sub>) values for *B. anthracis* Ames after aerosol inoculation. A/J and C57BL/6J mice were comparably susceptible to *B. cereus* G9241 by both subcutaneous and intranasal routes of infection. However, the LD<sub>50</sub>s for *B. cereus* G9241 in both mouse strains were markedly higher than those reported for *B. anthracis* Ames and more like those of the toxigenic but nonencapsulated *B. anthracis* Sterne. Furthermore, *B. cereus* G9241 spores could germinate and disseminate after intranasal inoculation into A/J mice, as indicated by the presence of vegetative cells in the spleen and blood of animals 48 h after infection. Lastly, *B. cereus* G9241 derivatives cured of one or both megaplasms were highly attenuated in A/J mice. We conclude that the presence of the toxin- and capsule-encoding plasmids pBCXO1 and pBC218 in *B. cereus* G9241 alone is insufficient to render the strain as virulent as *B. anthracis* Ames. However, like *B. anthracis*, full virulence of *B. cereus* G9241 for mice requires the presence of both plasmids.

The *Bacillus cereus* group, of which *Bacillus anthracis*, *Bacillus thuringiensis*, and *B. cereus* are members, are Gram-positive, spore-forming, rod-shaped bacteria that reside in the soil. The most pathogenic of the species toward humans is *B. anthracis*, the etiologic agent of inhalational, cutaneous, and gastrointestinal anthrax. *B. thuringiensis* is an insect pathogen that is used worldwide as a pesticide. However, in rare cases, *B. thuringiensis* has been identified as the etiological agent in cases of food poisoning, wound infection, and catheter-associated bacteremia (11, 20, 24). *B. cereus* is primarily associated with food-borne illness, and these cases usually arise from improper food preparation and storage. In addition, *B. cereus* is an opportunistic human pathogen that has been implicated in wound infections, endocarditis, osteomyelitis, endophthalmitis, and urinary tract infections in humans (2). Strains of *B. cereus* have also been associated with more severe “inhalational anthrax-like” infections in humans (12, 14, 15, 25, 37, 38) and chimpanzees (18). The abundance and distribution of *B. cereus* isolates that cause severe inhalational disease in humans are not clear, in part due to the genetic similarity among members of the *B. cereus* group. One of these *B. anthracis*-like *B. cereus*

strains, G9241, was isolated from a welder in Louisiana who survived a life-threatening, inhalational anthrax-like disease (14).

Members of the *B. cereus* group are closely related phylogenetically and can retain megaplasms that encode virulence genes (30). The fully virulent *B. anthracis* Ames strain contains the 189-kb pXO1 and the 96-kb pXO2, and both are required for full virulence (4, 41). The megaplasmid pXO1 contains the genes that encode the toxin components protective antigen (PA), edema factor (EF), and lethal factor (LF). The operon required for synthesis of the unique poly-γ-D-glutamic acid capsule is encoded on pXO2. The widely used laboratory strain *B. anthracis* Sterne contains pXO1 but not pXO2. *B. anthracis* Sterne is avirulent in rabbits (34), unlike *B. anthracis* Ames (34), but is of moderate but lower virulence than a pXO1- and pXO2-containing *B. anthracis* strain in most mouse models of anthrax disease (39, 41). *B. anthracis* Sterne is used as a live spore vaccine for cattle, and a similar attenuated derivative of wild-type *B. anthracis* was used as a vaccine in humans in the former Soviet Union (19, 34).

Megaplasms with high similarity to pXO1 and, in some cases, pXO2, have been identified in *B. cereus* isolates from pulmonary anthrax-like disease (12, 14, 18). *B. cereus* G9241 contains three endogenous plasmids: the megaplasms pBCXO1 and pBC218 and the smaller pBClin29. The plasmid pBCXO1 is >99% similar to pXO1 (14). The genes that encode PA (*pag*), EF (*cya*), and LF (*lef*) are all present on pBCXO1; the gene products are 99.7% (PA), 96% (EF), and 99% (LF) identical to the *B. anthracis* proteins. In addition,

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homologs of *pag* and *lef* are present on pBC218; however, the pBC218-encoded PA and LF are only 60% and 36% identical to their *B. anthracis* homologs (14). In addition, the pBC218-encoded LF does not contain the zinc metalloprotease domain. Fieldhouse et al. recently used computational models to propose that the pBC218-encoded LF, which they named Certhrax, contains a domain that is predicted to have ADP-ribosyltransferase activity; however, no experimental evidence of activity was presented (8). (Note that the plasmid pBC218 name is derived from its originally reported size of ~218 kb [14]; however, subsequent studies have shown that it is 210 kb, and it is thus designated pBC210 [31]. In this report we will refer to this plasmid by its original designation, pBC218.) While pBC218 is not similar in sequence to the capsule-encoding pXO2, pBC218 encodes a putative polysaccharide capsule operon, and *B. cereus* G9241 has been shown to produce a capsule (14, 36). The operon contains all of the genes that are predicted to encode the glycosyltransferases, translocase, polymerase, and regulatory elements required for polysaccharide capsule biosynthesis in Gram-positive bacteria (14). The third plasmid, pBClin29, contains genes that encode putative phage proteins (14). In *B. anthracis*, the toxin-encoding genes and the capsule operon are positively regulated by the pXO1-encoded *AtxA*; expression of *atxA* is increased in the presence of CO<sub>2</sub> (29). Homologs of *atxA* are found on both pBCXO1 and pBC218. Furthermore, Passalacqua et al. found that expression of the pBCXO1 *atxA* gene was upregulated in CO<sub>2</sub>, as was the putative pBC218 polysaccharide capsule operon (28).

*B. anthracis* has been used as a bioweapon in the United States and elsewhere (9, 16, 17). Because *B. cereus* G9241 and *B. anthracis* share phenotypic traits and can cause similar diseases in humans, we assessed the virulence of *B. cereus* G9241 in well-characterized animal models of *B. anthracis* infection (New Zealand White rabbits and A/J and C57BL/6J mice). Moreover, since both the toxin- and capsule-encoding plasmids (i.e., pXO1 and pXO2) are required for full virulence of *B. anthracis*, we generated plasmid-cured strains of *B. cereus* G9241 and assessed the contribution of each plasmid toward virulence, toxin production, and polysaccharide capsule production. Taken together, the data presented here demonstrate that, as with *B. anthracis*, both toxin expression and capsule production are required for full virulence of *B. cereus* G9241. However, we conclude that the presence of these *B. anthracis*-like plasmids is not sufficient to produce a strain of *B. cereus* that is as virulent as wild-type *B. anthracis* in rabbits or mice.

#### MATERIALS AND METHODS

**Bacteria strains and spore preparation.** All strains used in this work are listed in Table 1. Bacteria were routinely cultured in brain heart infusion (BHI) broth (BD Biosciences, San Jose, CA) at 35 to 37°C with aeration. Spores were prepared for rabbit challenge experiments by the shaker flask method (3), and spores for the mouse challenge experiments were produced on solid sporulation medium (26) as previously described (6, 33).

**Rabbit challenge models.** Because the virulence of *B. cereus* G9241 had not yet been established when these studies were initiated, all rabbit experiments were performed with animal biosafety level 3 (ABSL-3) containment in accordance with the Battelle Biomedical Research Center Institutional Animal Care and Use Committee regulations. Rabbit virulence was determined in specific-pathogen-free New Zealand White rabbits (Covance, Inc., Princeton, NJ) that were individually housed and ear tagged for identification. Rabbits (50% male, 50% female) weighed 2.3 to 3.5 kg at the beginning of the study. Six animals each were inoculated subcutaneously with ~10<sup>4</sup> or ~10<sup>5</sup> *B. cereus* G9241 spores (10 or 100

TABLE 1. Bacterial strains used in this study

Species and strain	Plasmid(s) present	Source or reference
<i>B. anthracis</i> Sterne 34F2	pXO1	35
<i>B. cereus</i> G9241	pBCXO1, pBC218, and pBClin29	14
pBCXO1 <sup>+</sup> /pBC218 <sup>-</sup>	pBCXO1 and pBClin29	This study
pBCXO1 <sup>-</sup> /pBC218 <sup>+</sup>	pBC218 and pBClin29	This study
pBCXO1 <sup>+</sup> /pBC218 <sup>-</sup>	pBClin29	This study
569 UM20	None	10

times the reported *B. anthracis* Ames 50% lethal dose [LD<sub>50</sub>] of 1.6 × 10<sup>3</sup> spores for rabbits inoculated subcutaneously [43]). Two rabbits were challenged with ~10<sup>4</sup>, three rabbits with ~10<sup>5</sup>, and two rabbits with ~10<sup>7</sup> *B. cereus* G9241 spores by aerosol exposure (0.1 to 100 times the published *B. anthracis* Ames LD<sub>50</sub> of 1.1 × 10<sup>5</sup> spores for rabbits challenged by an inhalational route [43]). A modified Microbiological Research Establishment type three jet collision nebulizer (BGI, Waltham, MA) with a precious-well fluid jar was used to generate controlled delivery of an aerosol of spores in a water suspension. Rabbits were placed into a muzzle-only inhalation exposure chamber and subjected to *B. cereus* G9241 spores for 10 to 20 min. Rabbits were monitored twice daily for 14 days postexposure for morbidity, respiratory distress, and changes in appetite, activity, and temperature. In addition, blood was collected daily to assess bacteremia.

**Mouse challenge models.** All mouse experiments were conducted after rabbit data demonstrated that BSL-3 containment was unnecessary; as such, mouse studies were done under ABSL-2 conditions in accordance with the Uniformed Services University Institutional Animal Care and Use Committee regulations. Six-week-old female A/J and C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were housed in filter-top cages and provided food and water *ad libitum*. Spores were diluted to the desired dose in sterile water and inoculated into mice either intranasally or subcutaneously. For intranasal inoculation, mice were lightly anesthetized with isoflurane delivered through the XGI-8 gas anesthesia system (Caliper Life Sciences, Hopkinton, MA). Animals were then held upright, and 20 to 25 μl of a spore suspension or water was introduced directly onto both nares. Mice were held upright until the inoculum was inhaled. For subcutaneous inoculation, mice were injected behind the right foreleg with 0.1 ml spores diluted in sterile water or water alone. Mice were monitored daily for morbidity and mortality for 14 days postinoculation. The LD<sub>50</sub> and 95% confidence limits were calculated by probit analysis with SAS version 9.2 when permitted by the data; otherwise, the LD<sub>50</sub> was calculated by using Reed-Muench analysis (32).

**For *B. cereus* dissemination studies,** A/J mice were inoculated intranasally with 3 × 10<sup>6</sup> *B. cereus* G9241 spores (10 × the LD<sub>50</sub>) in 25 to 50 μl sterile water. To assess dissemination, mice (*n* = 5 per time point) were sacrificed 6, 24, and 48 h postinoculation, and organs (lungs, spleen, and heart) were collected. Blood was collected from the tail vein of mice prior to sacrifice. Organs were also collected from a group of five mice that succumbed naturally to infection. Organs were homogenized in 1 ml phosphate-buffered saline (PBS) with a tissue homogenizer (Omni International, Kennesaw, GA). Half of each blood or tissue sample was heat treated at 65°C to kill vegetative cells. All samples were serially diluted and plated for bacterial enumeration. Vegetative CFU/ml was calculated by subtraction of the spore CFU/ml (heat treated) from the total CFU/ml (not heat treated). The dissemination experiment was performed twice.

**Generation of plasmid-cured *B. cereus* G9241 strains.** Strains were cured of pBCXO1 and/or pBC218 by growth either in the presence of novobiocin or at 41°C by slight modifications of a previously published protocol (10). For generation of the pBCXO1<sup>+</sup>/pBC218<sup>-</sup> strain, a liquid culture of *B. cereus* G9241 was grown overnight in Luria-Bertani (LB) broth at 41°C with shaking at 225 rpm. On each of three consecutive days, the culture was diluted 1/1,000 into fresh LB broth and grown as above. Dilutions of the final culture were plated onto NBY-bicarbonate agar (nutrient broth, 8 g/liter; yeast extract, 3 g/liter; agar, 15 g/liter; 0.8% NaHCO<sub>3</sub>) and grown at 37°C in 5% CO<sub>2</sub> to promote capsule production. Individual colonies were passaged several times onto fresh NBY-bicarbonate plates and incubated as described above. Colonies with a rough appearance were isolated and tested for loss of pBC218 and presence of pBCXO1 by PCR as described below. For generation of the pBCXO1<sup>-</sup>/pBC218<sup>+</sup> strain, *B. cereus* G9241 was grown in LB broth supplemented with 2

TABLE 2. Sequences of plasmid test primers

Target	Gel lane	Amplified region (bp)	Primer pair sequences (5'→3')
Chromosome	1	<i>fabG</i> gene, GI 47564343	ATGAGATTGGCAAACGACGGTGCATTAG CTATAAACAAAACCTCCAGAAACATCTATAATCTG
pBClin29	2	19026–20219	GTGCATGAAATGATTTTAGGTACGGAAAAACAGC TCAATTATCACCCAAGTACAATCTAGGTAGATTGGC
pBCXO1	3	16930–18119	GGAGGTCCATAGAATAATATAGAACGGGATGC CAGCACTATCAACACTGGAGCGATTTC
	4	32346–46593	TATCAAACAGATGTCAACCGTATCG CAGCATCTTTAACCTAGACCTAAC
	5	46593–47364	CTACATTGCTGACATTCAAAGGTAG GTACGGTGATACAACACAAAATTGAC
	6	61760–62583	GTTTGCCAGCTCCATTCCCAATAAG GGCTGAAAGCGTTAAGGCATATTCC
	7	77380–78264	CCTTCTGCGGTGTACTTGTTAATGG AATGCCTGACGGCACAACAATGTTC
	8	93388–94239	TACGTGATTACGACGACATAGTAG TACAGCGATACCAGATACTCTCTGTC
	9	123095–124127	AAGGTTTCATCTTCAGGCACAGATTTC CAGCTCTTGATCCACTATAGGATTTC
	10	139725–140879	CTTCTACAGTCACTTGGTCTCTAGTGAGTGGAC CATCCATTGAATTAGGTTTCATCTTGGCATATGGATC
	11	155955–156946	GTACAAGAGGAAGAGCAAGTAAATC CTCATTCCCATTCTTCTCTTATC
	12	170672–171559	GTGAAGATTAGAAAAGCGATTATCCCAGCAG TTATTTATTGATAAGTTTATCTCGAGTGATATTTCTCAAATAACTC
pBC218	13	14756–15936	GTTACGCAGCATCTCGAATATTAAC AAATCTTGCCCATGTCCAATAAGG
	14	29999–30609	CATCATATCTGCTATATAAGCCCCATGTA GTTGGAACTGGATCAGATGAGATTTAAGG
	15	44761–45728	CGACTAAATTGCTGAAGGCAGTAAC TTAGGAGGAAGGTAAATGAGGAAAAG
	16	60017–60948	CGCTTATATGCCAATATCCCTAATG AAATCTCTTTCGGTAGGGAAATCTG
	17	83364–84403	ACCCACTATATTCGGACTAAATACC ATGACCTTTGGGCACAGTATGTAAC
	18	107041–107811	ATTCTCTCGTCTCCCTCCTGATTTC GCACGGGACTTTCCGATAGACATGC
	19	123026–123817	ATACATACCGTGAATTCGTGTTCTC TAGGTGTTTATGGGACTACGGTTAC
	20	139043–139856	ACCGATCAACTCCTCAAGAACTGATCC TCCGGCTTCAACCTATTCCATCTTCAGTC
	21	155428–156313	CAGTATTCGTGCTAAAGGTTATAGG ACCGGTAATATCCAATCATTAGGTG
	22	171874–172635	AGCGTGCTGTAAGGTTGTTAGAATC CATTTGTCTTCATCAGGTCAATAGG
	23	187803–189013	ATTATCAACCACGAGTGGTACGTAG AGGATTTTCGGTGTTTAATAGCTGAC
	24	208076–208938	GTATTAGCGAAATGAGACGGAGAAG CTTTGTAATTTCTCCACCGACAGAC

μg/ml novobiocin at 37°C with shaking at 225 rpm for 3 days. Dilutions were plated as described above. Smooth colonies were streaked for isolation and screened by PCR for loss of pBCXO1 and maintenance of pBC218. The pBCXO1<sup>+</sup>/pBC218<sup>+</sup> strain was directly derived from the pBCXO1<sup>+</sup>/pBC218<sup>+</sup> strain by the same novobiocin method except that rough colonies were screened by PCR.

For PCR screening of candidate strains, individual colonies were lysed in sterile, nuclease-free water at 98°C for 10 min. The lysate was used as the template for PCRs that included GoTaq Green master mix (Promega, Madison, WI) and a primer set unique to different regions on each of the *B. cereus* G9241 plasmids pBCXO1 and pBC218 (Table 2). Control reactions designed to amplify a region of the *B. cereus* G9241 chromosome and pBClin29 plasmid were included. PCR assays were done in either a Tetrad 2 (Bio-Rad) or a PTC-200 (MJ Research, Waltham, MA) thermocycler under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 1 min, 61°C for 1 min, and 72°C for 1.5 min; 72°C final

extension for 7 min. For primers pBC218-14 and pBC218-18, the annealing temperature was increased to 65°C to prevent nonspecific product formation. The initial PCR screen consisted of only one primer set for each plasmid to minimize reagent consumption and maximize screening throughput. Once candidate clones were obtained, they were further screened with the full panel of primers (Table 2). PCR products were separated by 1% agarose gel electrophoresis, and intercalated ethidium bromide was visualized with UV light.

**Phenotypic characterization of *B. cereus* G9241 plasmid-cured strains.** The growth rates of each derivative of *B. cereus* G9241 and *B. anthracis* Sterne in BHI broth were determined in triplicate according to standard protocols. To assess germination rates of the *B. cereus* G9241 derivatives and *B. anthracis* Sterne, the decrease in optical density at 562 nm (OD<sub>562</sub>) of triplicate cultures was followed over time in an EL800 absorbance microplate reader (BioTek, Winooski, VT). Spores were diluted to either the same OD<sub>562</sub> (≈1.0) or to the same final CFU/ml (1 × 10<sup>7</sup> CFU/ml), and germination was initiated with 100 μM inosine

TABLE 3. Virulence of *B. anthracis* Ames, *B. anthracis* Sterne, and *B. cereus* G9241 wild-type and plasmid-cured derivatives in mice

Mouse strain	Bacterial strain	Subcutaneous LD <sub>50</sub> <sup>a</sup>	Source(s)	Intranasal LD <sub>50</sub> <sup>a</sup>	Source(s)
A/J	<i>B. anthracis</i>				
	Ames (pXO1 <sup>+</sup> /pXO2 <sup>+</sup> )	0.7	21	3.8	21
	Sterne (pXO1 <sup>+</sup> /pXO2 <sup>-</sup> )	3.2	5, 33, 41	4.8	5, 33
	<i>B. cereus</i> G9241				
	pBCXO1 <sup>+</sup> /pBC218 <sup>+</sup>	3.1 (2.8–3.4) <sup>b</sup>	This study	5.5 <sup>c</sup>	This study
	pBCXO1 <sup>+</sup> /pBC218 <sup>-</sup>	6.8 (6.5–7.2) <sup>b</sup>	This study	>7	This study
C57BL/6J	<i>B. anthracis</i>				
	Ames (pXO1 <sup>+</sup> /pXO2 <sup>+</sup> )	1.4	4, 21	4.1	21
	Sterne (pXO1 <sup>+</sup> /pXO2 <sup>-</sup> )	5.9	41	>7	This study
	<i>B. cereus</i> G9241				
	pBCXO1 <sup>+</sup> /pBC218 <sup>+</sup>	3.7 (3.3–4.1) <sup>b</sup>	This study	5.8 (5.4–6.2) <sup>b</sup>	This study

<sup>a</sup> LD<sub>50</sub> values are the log<sub>10</sub> CFU, with 95% confidence intervals indicated in parentheses.

<sup>b</sup> LD<sub>50</sub> values and confidence intervals were determined with the probit method.

<sup>c</sup> The LD<sub>50</sub> value was determined with the Reed-Muench method (32), as these data did not permit analysis by probit.

and 50  $\mu$ M L-alanine. The ratio (OD<sub>initial</sub> – OD<sub>time</sub>)/(OD<sub>final</sub> – OD<sub>initial</sub>) was plotted versus time, and the germination rate was calculated from the slope of the linear portion of the curve (1, 23).

For detection of the toxin subunits PA, EF, and LF, overnight HI cultures of each strain were diluted 1/10,000 into HI broth that contained 0.8% sodium bicarbonate and grown at 37°C in 5% CO<sub>2</sub> for 24 h. Each bacterial culture was pelleted by centrifugation, and culture supernatants were further clarified by vacuum filtration with 0.22- $\mu$ m cellulose acetate filters (Corning, Acton, MA). Volumes of cleared supernatant were normalized prior to concentration based on the final OD<sub>600</sub> of the culture. Filtrates were concentrated with Amicon Ultra-4 10,000 MWCO regenerated cellulose centrifugal filter units (Millipore, Billerica, MA). Toxin subunits in culture supernatant filtrates were detected by Western blotting. Recombinant PA, LF, or EF (List Biologicals, Campbell, CA) and *B. anthracis* Sterne culture filtrate served as positive controls for Western blot assays. Culture filtrate of *B. cereus* 569 UM20 (10), a nontoxicogenic and nonencapsulated *B. cereus* strain, served as a negative control for Western blot assays. The PA and LF blots were blocked with 5% skim milk (SM) and 0.1% Tween 20 (Fisher Scientific, Pittsburgh, PA) in PBS (PBST), and the EF blot was blocked with 5% normal rabbit serum in protein-free T20 blocking buffer (Thermo Fisher Scientific, Rockford, IL). Rabbit polyclonal anti-PA antibodies (Abcam, Cambridge, MA) were diluted 1/1,000 in SM-PBST, mouse monoclonal anti-PA and mouse monoclonal anti-LF (BEI Resources, Manassas, VA) were diluted 1/10,000 in SM-PBST, and goat polyclonal anti-EF (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1/1,000 in protein-free T20 buffer. The secondary antibodies goat anti-rabbit Ig-horseradish peroxidase (HRP; 1/10,000), goat anti-mouse Ig-HRP (1/10,000), and rabbit anti-goat Ig-HRP (1/20,000; Bio-Rad Laboratories, Hercules, CA) were diluted in PBST. The ECL Plus Western blotting detection system (GE Healthcare, Fairfield, CT) and X-Omat film (Kodak, Rochester, NY) were used to visualize the toxin subunits.

## RESULTS

***B. cereus* G9241 spores are nearly avirulent in rabbits but evoke lethal anthrax-like disease in mice.** In preliminary virulence studies, Hoffmaster et al. demonstrated that intraperitoneal inoculation of A/J mice with 10<sup>4</sup> or 10<sup>6</sup> *B. cereus* G9241 spores or *B. anthracis* Sterne spores caused 100% lethality (14). To more thoroughly assess the virulence of *B. cereus* G9241, we inoculated groups of New Zealand White rabbits with *B. cereus* G9241 at doses that ranged from 0.1 to 100 times the reported LD<sub>50</sub> for *B. anthracis* Ames delivered via subcutaneous (1.6  $\times$  10<sup>3</sup> CFU) or inhalational (1.1  $\times$  10<sup>5</sup> CFU) routes (43). Of the rabbits inoculated with *B. cereus* G9241 spores, all animals survived subcutaneous challenge (12/12) and 6/7 survived aerosol exposure; the rabbit that succumbed to infection was inoculated with  $\sim$ 10<sup>7</sup> spores.

To further investigate the virulence of *B. cereus* G9241, we inoculated A/J and C57BL/6J mice with various doses of *B. cereus* G9241 via intranasal and subcutaneous routes. The LD<sub>50</sub> for *B. cereus* G9241 delivered intranasally was calculated to be 3.2  $\times$  10<sup>5</sup> spores for A/J mice and 6.3  $\times$  10<sup>5</sup> spores for C57BL/6J mice (Table 3). Each of these LD<sub>50</sub>s is about 50-fold higher than the reported LD<sub>50</sub> for *B. anthracis* Ames in these strains of mice (21). While the LD<sub>50</sub>s for *B. cereus* G9241 were similar in both strains of mice, the median times to death for C57BL/6J mice inoculated intranasally with 10<sup>6</sup> or 10<sup>7</sup> spores of *B. cereus* G9241 were significantly longer than for A/J mice (Fig. 1A). The LD<sub>50</sub>s for *B. cereus* G9241 administered subcutaneously were calculated to be 1.3  $\times$  10<sup>3</sup> spores for A/J mice and 5.0  $\times$  10<sup>3</sup> spores for C57BL/6J mice (Table 3). These doses are approximately 400-fold (A/J) and 300-fold (C57BL/6J) higher than the reported LD<sub>50</sub>s for *B. anthracis* Ames (4, 21, 41). We observed that, as above for mice inoculated intranasally, C57BL/6J mice inoculated subcutaneously with *B. cereus* G9241 had a significantly longer median time to death than did A/J mice at a dose of 10<sup>4</sup> spores (Fig. 1B). Taken together, our data show that *B. cereus* G9241 is significantly attenuated compared to reported *B. anthracis* Ames LD<sub>50</sub> values in identical rabbit and mouse models of infection (4, 21, 41).

***B. cereus* G9241 disseminates from the lungs to the spleen.** Previous studies of mice showed that fully virulent *B. anthracis* disseminates from the lungs to distal organs during the course of acute infection (7, 21). To determine if *B. cereus* G9241 exhibits similar dissemination patterns, we challenged A/J mice intranasally with 3  $\times$  10<sup>6</sup> *B. cereus* G9241 spores (10 $\times$  the LD<sub>50</sub>) and enumerated both heat-sensitive (vegetative) and heat-resistant (spores) CFU from the lungs, spleen, and blood. We observed both spores and vegetative cells in the lungs within the first 6 h, a finding which suggests that germination occurred in the lungs (Fig. 2A). Both forms of *B. cereus* G9241 continued to be present in the lungs 24 and 48 h postinoculation in the majority of mice. No spores were detected in the blood or spleen at any of the times that we tested, but increasing numbers of vegetative bacilli were found in both compartments beginning 48 h postinoculation (Fig. 2B and C). In mice that succumbed to infection (open symbols in Fig. 2), we de-



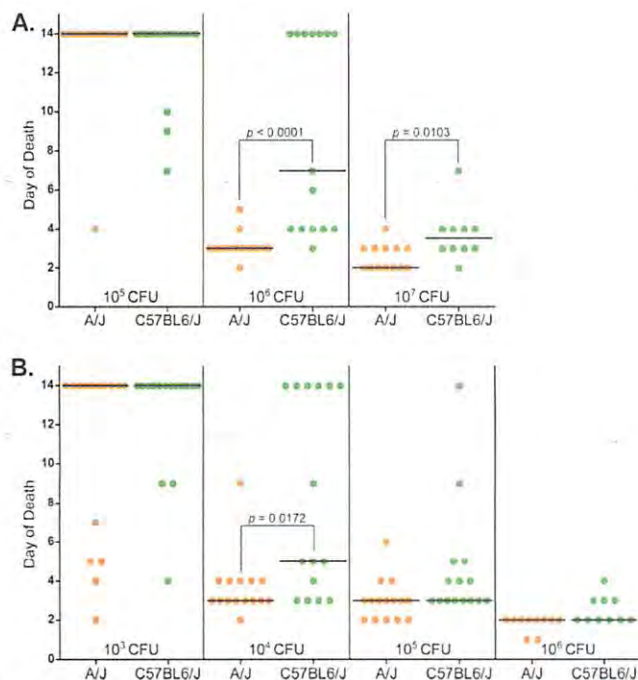


FIG. 1. Dose response of A/J and C57BL/6J mice to *B. cereus* G9241. A/J and C57BL/6J mice were inoculated by the intranasal (A) or subcutaneous (B) route with *B. cereus* G9241 spores to determine the LD<sub>50</sub> for each route. Each symbol (●) represents one A/J (orange) or one C57BL/6J (green) mouse. The median time to death at each dose is represented by the black horizontal line; a time to death of 14 days was assigned to all mice that survived the experiment. Significant differences in the median time to death were calculated with the Mann-Whitney rank sum test, and the *P* values are shown on the graph where applicable.

ected both spores and vegetative cells in the lungs, as well as high numbers of vegetative cells in the blood and spleen. In aggregate, these data demonstrate that spores germinate in the lungs early in infection and the resultant vegetative cells disseminate systemically within 48 h postchallenge.

**Plasmid-cured *B. cereus* G9241 strains are attenuated for virulence in mice.** To assess the involvement of the toxins and capsule in the virulence of *B. cereus* G9241, we cured *B. cereus* G9241 of either the toxin-encoding plasmid pBCXO1, the putative polysaccharide capsule operon-encoding plasmid pBC218, or both plasmids. We screened and confirmed megaplasmid loss in each of the cured strains by PCR (Fig. 3A). Thorough screening was required, because temperature and/or novobiocin treatment often resulted in only partial curing of the plasmids in which fragments/regions of the parent plasmid were detected by PCR (data not shown). Lastly, we determined that *B. cereus* G9241 and its plasmid-cured derivatives all displayed similar growth rates and germination rates *in vitro* (data not shown).

To determine whether the secreted toxin components PA, EF, and LF were produced by *B. cereus* G9241 and the plasmid-cured derivatives, we analyzed supernatants of cultures grown under toxin-inducing conditions for toxin antigens by immunoblotting. As shown in Fig. 3B, we detected PA, EF, and LF in culture supernatants from *B. anthracis* Sterne and from pBCXO1<sup>+</sup> *B. cereus* G9241 strains. However, the relative

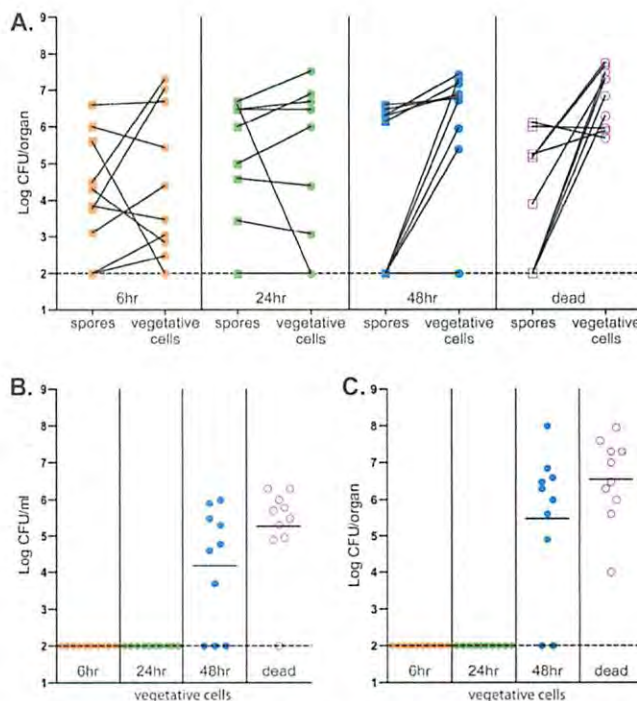


FIG. 2. *B. cereus* G9241 dissemination following intranasal inoculation. A/J mice were intranasally inoculated with  $3 \times 10^6$  *B. cereus* G9241 spores. Mice were sacrificed at 6 h (orange), 24 h (green), or 48 h (blue), or they succumbed to infection (purple). At each time point, lungs (A), blood (B), and spleen (C) were harvested, homogenized, and plated for bacterial enumeration with and without heat treatment. Spore CFU (■) were enumerated directly from the heat-treated samples, and vegetative CFU (●) were enumerated by subtraction of CFU in heat-treated samples from total CFU in non-heat-treated samples. Data shown are from two independent experiments ( $n = 5$  mice per group), with a total of 10 mice per time point, and each mouse is represented by an individual symbol. A solid black line connects the spore and vegetative CFU for each mouse in panel A. The horizontal lines in panels B and C represent the geometric means for each group, and the dashed lines in all panels are the limits of detection.

abundance of the three toxin subunits was lower in the pBCXO1<sup>+</sup>/pBC218<sup>-</sup> derivative than in the wild-type strain. In addition, we did not detect PA or LF in the pBCXO1<sup>-</sup>/pBC218<sup>+</sup> derivative despite the presence of *pag* and *lef* homologs on pBC218. None of the commercially available antibodies against PA from *B. anthracis* that were tested in this study were cross-reactive with recombinant pBC218-encoded PA (data not shown). The epitope recognized by the monoclonal anti-LF antibody is not predicted to be part of the truncated LF carried on pBC218 (8), so lack of reactivity with this antibody does not necessarily indicate lack of expression of the toxin subunit.

We stained vegetative bacilli grown under conditions that promote capsule production with India ink to qualitatively determine whether capsule was produced by each of the *B. cereus* G9241 strains (Fig. 3C). We observed a negatively stained halo indicative of capsule expression around both pBC218<sup>+</sup> strains and no halo around the pBC218<sup>-</sup> strains. These data provide experimental evidence that pBC218 is required for capsule production by *B. cereus* G9241.



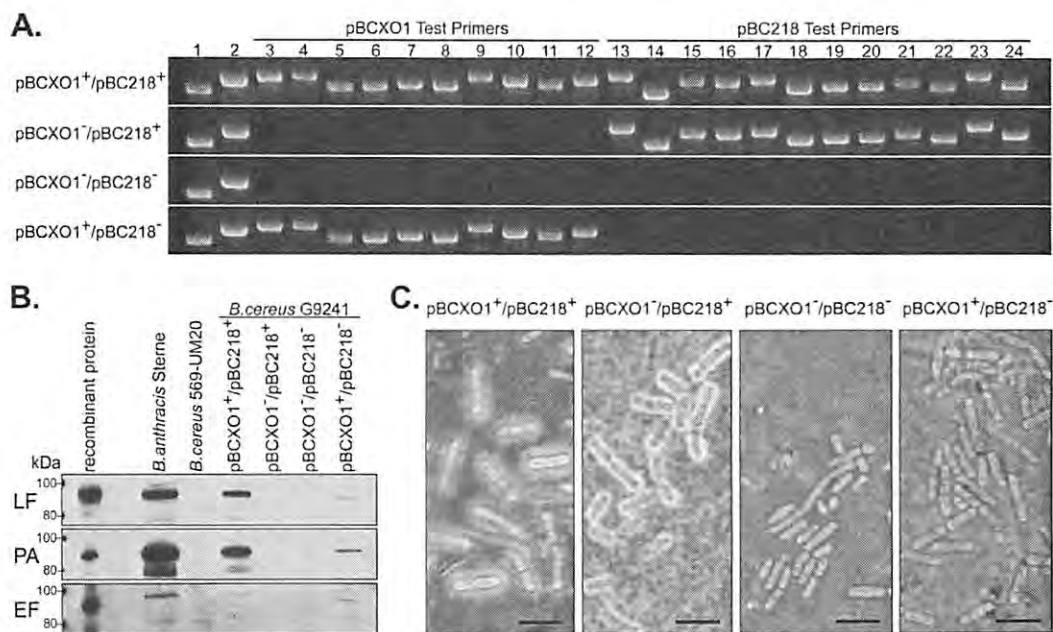


FIG. 3. Characterization of *B. cereus* G9241 cured strains. (A) PCR screening of the *B. cereus* G9241 strains was done with primers specific for regions on pBCXO1 (lanes 3 to 12) and pBC218 (lanes 13 to 24) to verify the presence or absence of these plasmids; lysed bacterial cells were used as the PCR template. Primers for chromosomal DNA (lane 1) and pBClin29 (lane 2) were included as controls. (B) Western blot analysis of supernatants from cultures grown under toxin-inducing conditions. Recombinant *B. anthracis* toxin subunit proteins and *B. anthracis* Sterne culture supernatants were included as positive controls, and *B. cereus* 569 UM20 culture supernatant was a negative control. Cultures were normalized to the same OD<sub>600</sub> value prior to concentration of the culture supernatant. (C) India ink stain of the *B. cereus* G9241 strains. Bar, 5  $\mu$ m. A negatively stained halo around the bacteria is indicative of capsule expression.

To assess the virulence of the *B. cereus* G9241 plasmid-cured derivatives, we inoculated A/J mice via intranasal and subcutaneous routes with various doses of spores from the plasmid-cured strains. Of all the plasmid-cured strains, only the *B. cereus* G9241 pBCXO1<sup>-</sup>/pBC218<sup>-</sup> derivative caused mortality after subcutaneous inoculation at a dose >3 logs higher than the *B. cereus* G9241 LD<sub>50</sub> (Table 3). These results indicate that both the polysaccharide capsule and the toxins are necessary for full virulence of *B. cereus* G9241 in these murine models of anthrax-like disease.

## DISCUSSION

In this study, we showed that *B. cereus* G9241 is attenuated for virulence in rabbits and mice compared to published data for *B. anthracis* Ames, despite the facts that *B. cereus* G9241 was isolated from a patient who survived an inhalational anthrax-like disease and that the strain possesses virulence plasmids analogous to those of *B. anthracis* Ames. In our hands, *B. cereus* G9241 was avirulent when delivered to New Zealand White rabbits via subcutaneous injection. When delivered via aerosol droplets, the primary mechanism by which *B. anthracis* spores are introduced into the human airway, *B. cereus* G9241 was nearly avirulent and required a dose approximately 100 $\times$  the LD<sub>50</sub> for *B. anthracis* Ames before the sole death was observed. In both A/J and C57BL/6J mice, *B. cereus* G9241 caused lethal anthrax-like disease after subcutaneous and intranasal inoculation. However, the number of *B. cereus* G9241 spores that caused 50% mortality was 50 times and 300 to 400 times greater than the LD<sub>50</sub>s for *B. anthracis*

Ames delivered intranasally and subcutaneously, respectively (4, 21, 41). Therefore, we conclude that *B. cereus* G9241 is significantly less virulent than wild-type *B. anthracis* in both strains of mice. This conclusion is further supported by comparison of the virulence of *B. cereus* G9241 and *B. anthracis* Sterne, an attenuated strain of *B. anthracis* that lacks pXO2 and is nonpathogenic toward humans. In A/J mice, the LD<sub>50</sub> for *B. cereus* G9241 is similar for mice inoculated subcutaneously and only approximately 5 times greater for those inoculated intranasally than the reported LD<sub>50</sub> values for *B. anthracis* Sterne (5, 33, 41). Since the pathological features of anthrax disease in humans are well mimicked in rabbit infection models and A/J and C57BL/6J mouse models indicate decreased susceptibility relative to virulent *B. anthracis* Ames, we conclude that *B. cereus* G9241 is less virulent than *B. anthracis* Ames.

One similarity between *B. cereus* G9241 and *B. anthracis* Ames is the presence of a capsule surrounding the bacterium that is known in the case of Ames to protect the organism from opsonophagocytosis (22). C57BL/6J mice are more resistant than A/J mice to nonencapsulated *B. anthracis* strains, such as *B. anthracis* Sterne (41). The increased resistance has been attributed in part to the fact that C57BL/6J mice have an intact complement system, while A/J mice are C5 deficient (41). The C5 deficiency in A/J mice causes reduced recruitment of neutrophils and macrophages to the site of infection, which allows the spores to germinate and the bacilli to replicate and disseminate more efficiently. The protective effect of the capsule for *B. cereus* G9241 is suggested by the slight increase in LD<sub>50</sub> values of approximately 2 $\times$  for intranasal and 4 $\times$  for subcutaneous routes of inoculation in C57BL/6J mice compared to

A/J mice (Table 3) and a statistically significant difference in the median time to death between the two mouse strains (Fig. 1); a similar modest increase in the LD<sub>50</sub> is also observed with *B. anthracis* Ames (4, 21, 41). In contrast, the LD<sub>50</sub> values for the nonencapsulated *B. anthracis* Sterne are ~150× and ~500× higher in C57BL/6J mice than in A/J mice inoculated via intranasal and subcutaneous routes, respectively (Table 3) (5, 33, 41). These observations suggest that the polysaccharide capsule of *B. cereus* G9241 may protect the organism from the host immune response, as does the polyglutamic acid capsule of *B. anthracis* Ames.

We also demonstrated that the megaplasmids pBCXO1 and pBC218 of *B. cereus* G9241 are required for full virulence of the organism in mice. In fact, curing of either plasmid rendered the organism nearly avirulent in A/J mice and curing of both plasmids resulted in complete avirulence. We showed that pBCXO1 is required for production of the anthrax toxin components PA, EF, and LF. However, we could not confirm with commercially available antibodies against *B. anthracis* PA and LF that the PA and LF genes carried on pBC218 were expressed under toxin-inducing conditions. Thus, the roles of the pBC218 PA and LF genes in the virulence of *B. cereus* G9241 remain unclear. In addition, we confirmed experimentally that pBC218 is required for capsule production; previous studies provided *in silico* but not *in vivo* evidence for a capsule biosynthetic operon on pBC218 (14, 36). Studies of the relative contributions of pXO1 and pXO2 to the pathogenesis of *B. anthracis* showed that strains that expressed pXO1 only caused lethality in immunocompromised mice and, to a lesser degree, in immunocompetent mice (41). In contrast, most strains that contained pXO2 only were as virulent as those that contained pXO1 and pXO2; the notable exception was *B. anthracis* Pasteur 6602 (pXO1<sup>-</sup>/pXO2<sup>+</sup>), which was avirulent in A/J mice (41, 42). Further, the chromosomal background in which pXO2 was expressed contributed significantly to the virulence of a *B. anthracis* strain (42). We attribute the reduced virulence of the plasmid-cured strains to the loss of toxins and/or capsule, which is analogous to the reduced virulence of *B. anthracis* Sterne (pXO1<sup>+</sup>/pXO2<sup>-</sup>) and *B. anthracis* Pasteur (pXO1<sup>-</sup>/pXO2<sup>+</sup>) compared to *B. anthracis* Ames (21); however, we acknowledge that additional factors carried on pBCXO1, pBC218, and/or the chromosome may contribute to the virulence of *B. cereus* G9241.

Neither of the single plasmid-cured strains produced wild-type levels of the toxin components or capsule, as determined by Western blotting (Fig. 3B) and India ink staining (Fig. 3C), respectively, despite the fact that growth rates of all of the *B. cereus* G9241 strains were similar. These data suggest that one or more positive regulators of virulence factor expression are encoded on pBCXO1 and pBC218. Hoffmaster et al. identified homologs of *atxA* and *pagR* on pBCXO1 and a homolog of *atxA* on pBC218 (14); both are encoded in *B. anthracis* on pXO1, and the expression of both genes is upregulated in the presence of CO<sub>2</sub>. The *B. anthracis* AtxA is a positive regulator of the toxin subunit genes *pagA*, *lef*, and *cya* (39) as well as the capsule operon (40), while PagR is a negative regulator of *pagA* and *atxA* (13). Passalacqua et al. demonstrated that the expression of the *B. cereus* G9241 pBCXO1 *atxA* is upregulated in the presence of CO<sub>2</sub> but the expression of the pBC218 *atxA* is unaffected by growth in O<sub>2</sub> or CO<sub>2</sub> (28). These results, taken

together with our findings in Fig. 3B, suggest that the absence of *atxA* carried on pBC218 is not the cause of reduced toxin production in the *B. cereus* G9241 pBCXO1<sup>+</sup>/pBC218<sup>-</sup> derivative and that additional pBC218-carried factors may be involved. Similarly, the pBCXO1<sup>-</sup>/pBC218<sup>+</sup> derivative exhibited reduced capsule production, as qualitatively observed by India ink staining (Fig. 3C); this observation suggests that the regulator(s) of capsule production, including *atxA*, may be carried on pBCXO1. Uchida et al. reported a similar reduction in capsule production by *B. anthracis* cured of pXO1 (40). Regulation of toxin and capsule expression in *B. cereus* G9241 is clearly multifactorial and likely involves other effectors that have yet to be described.

In conclusion, while *B. cereus* G9241 possesses two virulence plasmids required for toxin and capsule production that are analogous to those in the highly virulent *B. anthracis* Ames, our results strongly suggest that the virulence of *B. cereus* G9241 is similar to that of the attenuated vaccine strain *B. anthracis* Sterne. Furthermore, our data demonstrate that toxin production and encapsulation alone are not predictive of virulence in *Bacillus* organisms and that the individual species and genetic background are important determinants of the pathogenicity of any given *Bacillus* species. Lastly, our animal data and the human data from individuals with the potential for impaired lung function due to occupation (12, 14) imply that *B. cereus* G9241, like most *B. cereus* isolates, is an opportunistic rather than a frank pathogen.

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#### ADDENDUM

While the manuscript was under review, a study of the role of capsular polysaccharides in *B. cereus* G9241 pathogenesis was published that suggested a role for a second polysaccharide capsule operon encoded on pBCXO1 in the virulence of *B. cereus* G9241 (27).

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